Figs. 11 A ~~and B~~, 11B, and 11C are a flow diagram of a synthesis of derivatives of FK520 and chemical structures of FK520, where the bottom structures are designed to bind to mutant FKBP12.

Fig. 12 is a diagrammatic depiction of mutant FKBP with a modified FK520 in the putative cleft.

~~Fig. 13~~Figs. 13A and 13B ~~is~~are a flow diagram of the synthesis of heterodimers of FK520 and cyclosporin.

~~Fig. 16~~Figs. 16A and 16B depicts synthetic schemes for HED and HOD reagents based on FK-506-type moieties.

Fig. 17 depicts the synthesis of (CsA)2 beginning with CsA.

~~Fig 18~~Figs. 18A and 18B ~~is~~are an overview of the fusion cDNA construct and protien MZF3E.

~~Fig. 23~~Figs. 23A and 23B depicts the synthesis of modified FK-506 type compounds.

For the most part, for ease of construction, the transmembrane domain of the cytoplasmic domain or the receptor domain can be employed, which may tend to simplify the construction of the fused protein. However, for the lipid membrane retention domain, the processing signal will usually be added at the 5' end of the coding sequence for N-terminal binding to the membrane and, proximal to the 3' end for C-terminal binding. The lipid membrane retention domain will have a lipid of from about 12 to 24 carbon atoms, particularly 14 carbon atoms, more particularly myristoyl, joined to glycine. The signal sequence for the lipid binding domain is an N-terminal sequence and can be varied widely, usually having glycine at residue 2 and lysine or arginine at residue 7 (Kaplan, *et al.*, *Mol. Cell. Biol.* (1988) 8, 2435). Peptide sequences involving post-translational processing to provide for lipid membrane binding are described by Carr, *et al.*, *PNAS USA* (1988) 79, 6128; Aitken, *et al.*, *FEBS Lett.* (1982) 150, 314; Henderson, *et al.*, *PNAS USA* (1983) 80, 319; Schulz, *et al.*, *Virology* (1984), 123, 2131; Dellman, *et al.*, *Nature* (1985) 314, 374; and reviewed in *Ann. Rev. of Biochem.* (1988) 57, 69.

An amino acid sequence of interest includes the sequence M-G-S-S-K-S-K-P-K-D-P-S-Q-R (SEQ ID NO: 1). Various DNA sequences can be used to encode such sequence in the fused receptor protein.

As an illustration of "Bumped FK1012s" we prepared C10 acetamide and formamide derivatives of FK506. See Figure 16A and our report, Spencer et al, "Controlling Signal Transduction with Synthetic Ligands," *Science* **262** 5136 (1993): 1019-1024 for additional details concerning the syntheses of FK1012s A-C and FK506M. We chose to create two classes of bumped FK1012s: one with a bump at C10 and one at C9. The R- and S-isomers of the C10 acetamide and formamide of FK506 have been synthesized according to the reaction sequence in Figure 05B. These bumped derivatives have lost at least three orders of magnitude in their binding affinity towards FKBP12 (Figure ~~16B~~16A (panel B)). The affinities were determined by measuring the ability of the derivatives to inhibit FKBP12's rotamase activity.

An illustrative member of a second class of C9-bumped derivatives is the spiro-epoxide (depicted in Figure ~~16C~~16B (panel C)), which has been prepared by adaptation of known procedures. See e.g. Fisher et al, *J Org Chem* **56** 8(1991): 2900-7 and Edmunds et al, *Tet Lett* **32** 48 (1991):819-820. A particularly interesting series of C9 derivatives are characterized by their sp3 hybridization and reduced oxidation state at C9. Several such compounds have been synthesized according to the reactions shown in Figure 16C.

It should be appreciated that heterodimers (and other hetero-oligomerizers) must be constructed differently than the homodimers, at least for applications where homodimer contamination could adversely affect their successful use. One illustrative synthetic strategy developed to overcome this problem is outlined in Figure ~~16D~~16B (panel D). Coupling of mono alloc-protected 1,6-hexanediamine (Stahl et al, *J Org Chem* **43** 11 (1978): 2285-6) with a derivatized form of FK506 in methylene chloride with an excess of triethylamine gave an alloc-amine-substituted FK506 in 44% yield. This intermediate can now be used in the coupling with any activated FK506 (or bumped-FK506) molecule. Deprotection with catalytic tetrakis-triphenylphosphine palladium in the presence of dimedone at rt in THF removes the amine protecting group. Immediate treatment with an activated FK506 derivative, followed by

desilylation leads to a dimeric product. This technique has been used to synthesize the illustrated HOD and HED reagents.

A fourth example involves treatment of chronic pain with endorphin via encapsulation. A stock of human fibroblasts is transfected with a construct in which the chimeric transcriptional regulatory protein controls the transcription of human endorphin. The DNA construct consists of three copies of the binding site for the HNF-1* transcription factor GTTAAGTTAAC (SEQ ID NO: 2) upstream of a TATAAA site and a transcriptional initiation site. The endorphin cDNA would be inserted downstream of the initiation site and upstream of a polyadenylation and termination sequences. Optionally, the endorphin cDNA is outfitted with "PEST" sequences to make the protein unstable or AUUA sequences in the 3' nontranslated region of the mRNA to allow it to be degraded quickly.

The plasmid pSXNeo/IL2 (IL2-SX) (Fig. 1), which contains the placental secreted alkaline phosphatase gene under the control of human IL-2 promoter (-325 to +47; MCB(86) 6, 3042), and related plasmid variants (*i.e.* NFAT-SX, NFB-SX, OAP/Oct1-SX, and AP-1-SX) in which the reporter gene is under the transcriptional control of the minimal IL-2 promoter (-325 to -294 and -72 to +47) combined with synthetic oligomers containing various promoter elements (*i.e.* NFAT, NKB, OAP/Oct-1, and AP1, respectively), were made by three piece ligations of 1) pPL/SEAP (Berger, *et al.*, *Gene* (1988) 66,1) cut with SspI and HindIII; 2) pSV2/Neo (Southern and Berg, *J. Mol. Appl. Genet.* (1982) 1, 332) cut with NdeI, blunted with Klenow, then cut with PvuI; and 3) various promoter-containing plasmids (*i.e.* NFAT-CD8, B-CD8, cx12lacZ-Oct-1, AP1-LUCIF3H, or cx15IL2) (described below) cut with PvuI and HindIII. NFAT-CD8 contains 3 copies of the NFAT-binding site (-286 to -257; *Genes and Dev.* (1990) 4, 1823) and cx12lacZ-Oct contains 4 copies of the OAP/Oct-1/(ARRE-1) binding site (*MCB*, (1988) 8, 1715) from the human IL-2 enhancer; B-CD8 contains 3 copies of the NFB binding site from the murine light chain (*EMBO* (1990) 9, 4425) and AP1-LUCIF3H contains 5 copies of the AP-1 site (5'-TGACTCAGCGC-3' (SEQ ID NO: 3)) from the metallothionen promoter.

In vivo FK1012-induced protein dimerizati n We next wanted to confirm that intracellular aggregation of the MZF3E receptor is indeed induced by the FK1012. The influenza haemagglutinin epitope-tag (flu) of the MZF3E-construct was therefore exchanged with a different epitope-tag (flag-M2). The closely related chimeras, $MZF3E_{flu}$ and $MZF3E_{flag}$, were coexpressed in Jurkat T cells. Immunoprecipitation experiments using anti-Flag-antibodies coupled to agarose beads were performed after the cells were treated with FK1012A. In the presence of FK1012A (1μM) the protein chimera $MZF3E_{flag}$ interacts with $MZF3E_{flu}$ and is coimmunoprecipitated with $MZF3E_{flag}$. In absence of FK1012A, no coimmunoprecipitation of $MZF3E_{flu}$ is observed. Related experiments with FKBP monomer constructs $MZF1E_{flu}$ and $MZF1E_{flag}$, which do not signal, revealed that they are also dimerized by FK1012A (Figure 19A). This reflects the requirement for aggregation observed with both the endogenous T cell receptor and our artificial receptor MZF3E.

The various fragments were obtained by using primers described in Fig. 4 (SEQ ID NOS: 4, 6, 8, 10, 12, 14, 16, 17, 23, and 35). In referring to primer numbers, reference should be made to Fig. 4.

An approximately 1.2 kb cDNA fragment comprising the I-E chain of the murine class II MHC receptor (*Cell*, 32, 745) was used as a source of the signal peptide, employing P#6048 (SEQ ID NO: 4) and P#6049 (SEQ ID NO: 6) to give a 70 bp *Sac*II-*Xho*I fragment using PCR as described by the supplier (Promega). A second fragment was obtained using a plasmid comprising Tac (IL2 receptor chain) joined to the transmembrane and cytoplasmic domains of CD3 (*PNAS*, 88, 8905). Using P#6050 (SEQ ID NO: 8) and P#6051 (SEQ ID NO: 10), a 320 bp *Xho*I-*Eco*RI fragment was obtained by PCR comprising the transmembrane and cytoplasmic domains of CD3. These two fragments were ligated and inserted into a *Sac*II-*Eco*RI digested pBluescript (Stratagene) to provide plasmid, SPZ/KS.

To obtain the binding domain for FK506, plasmid rhFKBP (provided by S. Schreiber, *Nature* (1990) 346, 674) was used with P#6052 (SEQ ID NO: 33) and P#6053 (SEQ ID NO: 35) to obtain a 340 bp *Xho*I-*Sal*I fragment containing human FKBP12. This fragment was inserted into pBluescript digested with *Xho*I and *Sal*I to provide plasmid FK12/KS, which was the source

for the FKBP12 binding domain. SPZ/KS was digested with *Xho*I, phosphatased (cell intestinal alkaline phosphatase; CIP) to prevent self-annealing, and combined with a 10-fold molar excess of the *Xho*I-*Sal*I FKBP12-containing fragment from FK12/KS. Clones were isolated that contained monomers, dimers, and trimers of FKBP12 in the correct orientation. The clones 1FK1/KS, 1FK2/KS, and 1FK3/KS are comprised of in the direction of transcription; the signal peptide from the murine MHC class II gene I-E, a monomer, dimer or trimer, respectively, of human FKBP12, and the transmembrane and cytoplasmic portions of CD3. Lastly, the *Sac*II-*Eco*RI fragments were excised from pBluescript using restriction enzymes and ligated into the polylinker of pBJ5 digested with *Sac*II and *Eco*RI to create plasmids 1FK1/pBJ5, 1FK2/pBJ5, and 1FK3/pBJ5, respectively. See Figs. 3 and 4.

A myristoylation sequence from c-src was obtained from Pellman, *et al.*, *Nature* 314, 374, and joined to a complementary sequence of CD3 to provide a primer which was complementary to a sequence 3' of the transmembrane domain, namely P#8908 (SEQ ID NO: 23). This primer has a *Sac*II site adjacent to the 5' terminus and a *Xho*I sequence adjacent to the 3' terminus of the myristoylation sequence. The other primer P#8462 (SEQ ID NO: 12) has a *Sal*I recognition site 3' of the sequence complementary to the 3' terminus of CD3, a stop codon and an *Eco*RI recognition site. Using PCR, a 450 bp *Sac*II-*Eco*RI fragment was obtained, which was comprised of the myristoylation sequence and the CD3 sequence fused in the 5' to 3' direction. This fragment was ligated into *Sac*II/*Eco*RI-digested pBJ5(*Xho*I)(*Sal*I) and cloned, resulting in plasmid MZ/pBJ5. Lastly, MZ/pBJ5 was digested with *Sal*I, phosphatased, and combined with a 10-fold molar excess of the *Xho*I-*Sal*I FKBP12-containing fragment from FK12/KS and ligated. After cloning, the plasmids comprising the desired constructs having the myristoylation sequence, CD3 and FKBP12 multimers in the 5'-3' direction were isolated and verified as having the correct structure. See Figs. 2 and 4.

5' end of PCR amplified product:

```
        SacII              |----Gal4(1-147)--->>
                            M  K  L  L  S  S  I          (SEQ ID NO: 44)
5'      CGACACCGCGGCCACCATGAAGCTACTGTCTTCTATCG           (SEQ ID NO: 41)
```

Kozak

3' end of PCR amplified product:

```
        <<----Gal4(1-147----)|
        R  Q  L  T  V  S                    (SEQ ID NO: 46)
5'      GACAGTTGACTGTATCGGTCGACTGTCG        (SEQ ID NO: 45)
3'      CTGTCAACTGACATAGCCAGCTGACAGC        (SEQ ID NO: 77)
```

SalI

5' end of PCR amplified product:

```
SacII                |--HNF1(1-281)-->>
                        M  V  S  K  L  S          (SEQ ID NO. 50)
5'      CGACACCGCGGCCACCATGGTTTCTAAGCTGAGC        (SEQ ID NO. 49)
```

Kozak

3' end of PCR amplified product:

```
        <<-------HNF1 (1-282) ------|
        A  F  R  H  K  L                    (SEQ ID NO: 52)
5'      CCTTCCGGCACAAGTTGGTCGACTGTCG        (SEQ ID NO: 51)
3'      GGAAGGCCGTGTTCAACCAGCTGACAGC        (SEQ ID NO: 78)
```

SalI

Kozak

```
        M  L  E          (SEQ ID NO. 54)
5'  GGCCACCATGC          (SEQ ID NO. 53)
3'CGCCGGTGGTACGAGCT      (SEQ ID NO: 79)
```

*Sac*II overhang   *Xho*I overhang

```
                  T (ACN)
             126                    132
       L  D  P  K  K  K  R  K  V  L  E        (SEQ ID NO: 59)
5'     TCGACCCTAAGAAGAAGAGAAAGGTAC           (SEQ ID NO: 58)
3'         GGGATTCTTCTTCTCTTTCCATGAGCT       (SEQ ID NO: 80)

       SalI                      XhoI
```

```
           SalI   |--VP16(413-490)--->>
                   A  P  P  T  D  V          (SEQ ID NO: 64)
5'     CGACAGTCGACGCCCCCCCGACCGATGTC         (SEQ ID NO: 61)
```

3' end of PCR amplified product:

```
   <<--VP16(413-490)----|
      D  E  Y  G  G                          (SEQ ID NO: 66)
5'     GACGAGTACGGTGGGCTCGAGTGTCG            (SEQ ID NO: 65)
3'     CTGCTCATGCCACCCGAGCTCACAGC            (SEQ ID NO: 81)

                   Xho1
```

#37 38mer/0.2um/OFF 5'CGACACCGCGGCCACCATGAAGCTACTGTCTTCTATCG (SEQ ID NO: 41)

#38 28mer/0.2um/OFF 5'CGACAGTCGACCGATACAGTCAACTGTC (SEQ ID NO: 42)

#39 34mer/0.2um/OFF 5'CGACACCGCGGCCACCATGGTTTCTAAGCTGAGC (SEQ ID NO: 49)

#40 28mer/0.2um/OFF 5'CGACAGTCGACCAACTTGTGCCGGAAGG (SEQ ID NO: 48)

#43 29mer/0.2um/OFF 5'CGACAGTCGACGCCCCCCCGACCGATGTC (SEQ ID NO: 61)

#44 26mer/0.2um/OFF 5'CGACACTCGAGCCCACCGTACTCGTC (SEQ ID NO: 62)

#45 26mer/0.2um/OFF 5'GGCCACCATGC (SEQ ID NO: 53)

#46 18mer/0.2um/OFF 5'TCGAGCATGGTGGCCGC (SEQ ID NO: 55)

#47 27mer/0.2um/OFF 5'TCGACCCTAAGA-(C/A)-GAAGAGAAAGGTAC (SEQ ID NO: 56)

#48 27mer/0.2um/OFF 5'TCGAGTACCTTTCTCTTC-(G/T)-TCTTAGGG (SEQ ID NO: 57)

3. *Mutant hFKBP12 cDNA libraries* hFKBP12 may be digested with EcoRI and HindIII, blunted and cloned into pAS1 (Durfee et al, *supra*) that has been cut with NcoI and blunted. This plasmid is further digested with NdeI to eliminate the NdeI fragment between the NdeI site in the polylinker sequence of pAS1 and the 5' end of hFKBP12 and religated. This generated the hFKBP12-Gal4 DNA binding domain protein fusion. hFKBP was reamplified with primers #11206 (SEQ ID NO: 67) and #11210 (SEQ ID NO: 75), Primer Table:

Primer Table (SEQ ID NOS: 67-76): Primers used in the construction of a regionally localized hFKBP12 cDNA library for use in screening for compensatory mutations.

Although Applicants believe no fees other than those authorized for payment of the extension of time are due, if there are any fees due in connection with the filing of this Amendment, please charge the fees to our **Deposit Account No. 18-1945.** Please direct any questions arising from this submission to the undersigned at (617) 951-7739.

Respectfully Submitted,

Date: August 7, 2002

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